REMARKS

Claims 29-32 and 36-46 are pending in the application. As requested by the Examiner, Claims 31, 36 and 46 are amended to remove non-elected subject matter. Claim 29 is amended to define Applicants' invention with greater particularity. This amendment is fully supported by the specification and no new matter has been added. In view of the amendment and following remarks, Applicants respectfully request reconsideration of the application and claims and submit that the application is in condition for allowance.

I. Objections to the Disclosure and Claims

The Examiner objected to the disclosure because the Information Disclosure Statement references, R2 and R27, are missing the journal title, volume, and number on the accompanying PTO Form-1449. Applicants herewith submit a new PTO Form-1449 containing the requested information. Accordingly, Applicants respectfully request withdrawal of this objection. Applicants note that the Examiner appears not to have considered reference R7 on PTO Form-1449, i.e., Moss, M. and Frey. P.A., "The Role of S-Adenosylmethionine in the Lysine 2,3-Aminomutase Reaction," *The Journal of Biological Chemistry*, Vol. 262, No. 31, pp. 14859-14862 (1987). Applicants respectfully request the Examiner to consider reference R7 and initial PTO Form-1449.

The Examiner objected to Claims 31, 36, and 46 because they were both dependent from rejected Claims 30, 29, and 37, respectively, and because they are directed to non-elected subject matter. Applicants have mooted the second rejection by amending Claims 31, 36, and 46 to recite only the elected subject matter. In addition, as discussed below, Applicants believe amended Claim 29 and Claims 30 and 37 are patentable and, therefore, request that the Examiner withdraw the objections based on the rejection of these parent claims.

II. Rejections Under 35 U.S.C. § 112, second paragraph

In the Office Action, the Examiner rejected Claims 39, 41, and 45 under 35 U.S.C. § 112, second paragraph, as being indefinite. Claims 39 and 45 are directed to the method of producing L-ß-lysine of Claims 30 and 37, respectively. The Examiner argues that Claims 39 and 45 do not further limit Claims 30 and 37 because they recite cofactors inherently required for lysine 2,3-aminomutase activity. Applicants respectfully traverse this rejection.

The cofactors of Claims 39 and 45 are, in fact, merely individual species of the universe of possible cofactors. As the Examiner is no doubt aware, a cofactor is "a nonprotein component essential for the normal catalytic activity of an enzyme. Cofactors may be organic molecules ... or inorganic ions. They may activate the enzyme by altering its shape, or they may actually participate in the chemical reaction." *Concise Science Dictionary*, 2d, p. 145, (1991). Further, as the claims themselves show, more than one compound may serve the same role as a cofactor.

For example, both ferrous sulfate and ferric ammonium sulfate may serve as a source of iron for lysine 2,3-aminomutase. Similarly, dehydrolipoic acid, glutathione, or dithiothreitol may all serve as a source of reducing equivalents to maintain enzyme thiols in their reduced state. In each case, one of ordinary skill in the art would recognize that there are other compounds which could also serve as the required cofactors. For example, cysteine and dithioerythritol are also capable of maintaining thiols in their reduced state *in vitro*. See, e.g., Cleland, W. W., Biochemistry, 3, 480-482 (1964) (a copy of which is enclosed for the convenience of the Examiner). Therefore, one of ordinary skill in the art would appreciate that both cysteine and dithioerythritol could serve as one of the required cofactors for lysine 2,3-aminomutase and, thus, are embraced by parent Claims 30 and 37, but not by Claims 39 and 45. Other cofactors listed in Claims 39 and 45 also have equivalents that are embraced by the parent claims. It has long been known that dithionite can be replaced by mercaptoethanol-cobinamide system. Chirpich, T.P. et al., *J. Biol. Chem.*, 245, 1778-89, 1783 (1970) (included as R2 in Applicants' Form PTO-1449). It is also well known that S-

adenosylmethionine lacking a carboxyl group may be used in place of S-adenosylmethionine itself. Stadtman, T. in A. Meister, Ed., *Advances in Enzymology*, New York, 424, 413-447 (1973) (included as R27 in Applicants' Form PTO-1449). Therefore, Applicants respectfully submit that Claims 39 and 45 are not indefinite because they are distinguishable from their parent claims and ask that this rejection be withdrawn.

The rejection of Claim 41 as indefinite because of the lack of antecedent basis for "the immobilized lysine 2,3-aminomutase" in the parent claim is rendered moot by the current amendment. Applicants have amended Claim 41 to depend from Claim 40 directed to a method of producing L-ß-lysine using immobilized lysine 2,3-aminomutase. In addition, a typographical error in the claim was corrected. Applicants respectfully request that the rejection of claim 41 for indefiniteness be withdrawn.

III. Rejections Under 35 U.S.C. § 112, first paragraph

Claims 29, 30, 32, 37-45 are rejected under 35 U.S.C. § 112, first paragraph, "as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) at the time the application was filed had possession of the claimed invention." Office Action, page 6, lines 1-4. Although not using the terms, the Examiner appears to have imposed a written description and/or enablement rejection on the claims at issue. The Examiner goes on to argue there is a lack of representative species as encompassed by the Claims and "there is no disclosure of any particular structure to function/activity relationship in the disclosed species." Office Action, page 6, lines 14-15. Applicants respectfully disagree with this characterization of the invention.

In view of the present amendment, Applicants traverse the rejection of Claims 29, 30, 32, and 37-45 under 35 U.S.C. § 112, first paragraph. Claim 29 drawn to a method of producing L-ß-lysine, has been amended to recite the use of a prokaryotic host cell. The use of a prokaryotic host cell is clearly supported on page 9, lines 20-32, where a variety of prokaryotic hosts are disclosed, including E-coli, Clostridium, Haemophilius, and Bacillus

subtilus. Expression of proteins in prokaryotic hosts is well known to those skilled in the art and the specification incorporates by reference standard handbooks in the field such as *Molecular Biology Labfax* (Academic Press (1991)) and *DNA Cloning: A Practical Approach* (Glover (Ed.), IRL Press (1995)). Specification, page 9, lines 24-25, 30-32; page 47, line 5. In all, 34 strains of prokaryotic host cells are disclosed by the specification at page 9. Further, the specification discloses that an expression vector suitable for use in a prokaryotic cell may be comprised of transcriptional regulatory sequences such as promoters and enhancers, as well as translational regulatory sequences and a marker gene suitable for selection of cells that carry the expression vector. Specification, page 9, lines 4-7. The specification describes numerous promoters suitable for use in an expression vector (page 9, lines 8-16) and incorporates many more by reference such as those found in *Molecular Biology of the Gene*, 4d (Cummins, (1987)) Specification, page 9, lines 8-19. Further, the specification provides copious guidance as to the use of prokaryotic host cells and expression vectors in expressing proteins. Specification, page 10, lines 1-15. Finally, Examples 1-3 describe in detail how to use all of these techniques in identifying, claiming and expressing the Clostridial gene to produce lysine 2,3-aminomutase. Therefore, Claim 29 is well supported, enabled, and patentable.

Claim 30 is also definite and patentable because Applicants' disclosure teaches sufficient representative examples of lysine 2,3-aminomutase and methods to obtain any purified lysine 2,3-aminomutase. Applicants are not claiming all possible DNA sequences of lysine 2,3-aminomutase; rather, Applicants are claiming a method for producing L-ß-lysine which uses any purified lysine 2,3-aminomutase. Applicants' disclosure supports this claim because Applicants teach "the present invention contemplates the use of Clostridial enzyme sequences to identify lysine 2,3-aminomutase from other species. The present invention further contemplates variants of such lysine 2,3-aminomutases and the use of such enzymes to prepare ß-lysine." Specification, page 26, lines 18-21. Thus, Applicants expressly disclose that lysine 2,3-aminomutase genes can be obtained either by screening genomic or cDNA libraries or through the use of the polymerase chain reaction (PCR) with oligonucleotide primers that have nucleotide sequences "based upon the nucleotide sequences of the lysine 2,3-

aminomutase genes of Clostridium, Porphyromonas, Bacillus, Deinococcus, Aquifex, Teponema, Haemophilius or Escherichia, as described herein." *Id.*, page 26, lines 31-35. The specification discloses standard techniques can be used to identify and isolate these genes. See, e.g., specification, page 26, lines 25-30, line 35 - page 27, line 5.

Applicants have demonstrated that these techniques work even when the sequence identity in relation to the Clostridial enzyme is low. For example, the gene from E-coli has a sequence identity of only 31% to the corresponding Clostridial gene but was nonetheless identified using the Clostridial gene sequence. Specification, page 17, lines 9-23. Indeed, all of the other nucleotide sequences of lysine 2,3-aminomutase were identified based on the Clostridial gene. In addition, the specification discloses how to produce variants of Clostridial lysine 2,3-aminomutase containing conservative amino acid changes compared with the parent enzyme. Page 7, line 9 - page 8, line 30. One skilled in the art would understand that these methods may also be used to produce the variants of any lysine 2,3-aminomutase as contemplated on page 26, lines 19-21, of the application. Thus, the specification fully supports and enables a method of producing L-ß-lysine using any purified lysine 2,3-aminomutase. Hence, Claim 30 is well supported and enabled. Thus, Claims 32 and 37-45, which depend directly or indirectly from Claims 29 or 30, are also well supported and enabled. Applicants respectfully submit that Claims 29, 30, 32 and 37-45 are, therefore, patentable and request that the § 112, first paragraph rejections be withdrawn.

IV. Conclusion

In view of the foregoing remarks, Applicants request that the Examiner reconsider and withdraw the pending rejections discussed above. In the event any issues remain to be resolved in view of this communication, the Examiner is invited to contact the undersigned at the telephone number given below so that a prompt disposition of this application can be achieved.

Respectfully submitted,

Date April 25, 2003 By _____

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29. (Amended) A method of producing L-β-lysine, comprising:

(a) culturing a prokaryotic host cell comprising an expression vector that encodes lysine 2,3-aminomutase in the presence of L-lysine, wherein the cultured host cell expresses lysine 2,3-aminomutase, and

(b) isolating L-β-lysine from the cultured host cells.

31. (Amended) The method of claim 30, wherein the lysine 2,3-aminomutase has an amino acid sequence selected from the group consisting of (i) SEQ ID NO: 4, ~~(ii) SEQ ID NO:6, (iii) SEQ ID NO:8, (iv) SEQ ID NO:10, (v) SEQ ID NO:12, (vi) SEQ ID NO:14, (vii) SEQ ID NO:16, (viii) SEQ ID NO:2~~ and (iix) a conservative amino acid variant of ~~any of~~ SEQ ID NOs:~~2,~~ ~~4, 6, 8, 10, 12, 14, or 16~~.

36. (Amended) The method of claim 29 wherein the vector that encodes lysine 2,3-aminomutase has a nucleic acid sequence of~~selected from the group consisting of (i) SEQ ID NO: 1; (ii) (ii)~~ SEQ ID NO: 3~~, (iii) SEQ ID NO: 5, (iv) SEQ ID NO: 7, (v) SEQ ID NO: 9 , (vi) SEQ ID NO:11, (vii) SEQ ID NO:13, and (viii) SEQ ID NO:15~~.

41. (Amended) The method of claim ~~37~~40 wherein the L-lysine is contacted with the immobilized lysine 2,3-aminomutase for a sufficient amount of time to produce enantiomerically pure L-β-lysine.

46. (Amended) The method of claim 37, wherein the lysine 2,3-aminomutase has an amino acid sequence selected from the group consisting of (i) ~~SEQ ID NO:2, (ii)~~ SEQ ID NO: 4, ~~(iii) SEQ ID NO:6, (iv) SEQ ID NO:8, (v) SEQ ID NO:10, (vi) SEQ ID NO:12, (vii) SEQ ID NO:14, (viii) SEQ ID NO:16~~ and (iix) a conservative amino acid variant of ~~any of~~ SEQ ID NOs:~~2,~~ ~~4, 6, 8, 10, 12, 14, or 16~~.

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coenocyte A mass of protoplasm containing many nuclei and enclosed by a cell wall. It is found in certain algae and fungi. *Compare* cell; plasmodium; syncytium.

coenzyme An organic nonprotein molecule that associates with an enzyme molecule in catalysing biochemical reactions. Coenzymes usually participate in the substrate–enzyme interaction by donating or accepting certain chemical groups. Many vitamins are precursors of coenzymes. *See also* cofactor.

coenzyme A (CoA) A complex organic compound that acts in conjunction with enzymes involved in various biochemical reactions, notably the oxidation of pyruvate via the *Krebs cycle and fatty-acid oxidation and synthesis. It comprises principally the B vitamin *pantothenic acid, the nucleotide *adenine, and a ribose–phosphate group.

coenzyme Q (ubiquinone) Any of a group of related quinone-derived compounds that serve as electron carriers in the *electron transport chain reactions of cellular respiration. Coenzyme Q molecules have side chains of different lengths in different types of organisms but function in similar ways.

coercive force (coercivity) The magnetizing force necessary to reduce the flux density in a magnetic material to zero. *See* hysteresis.

cofactor A nonprotein component essential for the normal catalytic activity of an enzyme. Cofactors may be organic molecules (*coenzymes) or inorganic ions. They may activate the enzyme by altering its shape or they may actually participate in the chemical reaction.

coherent radiation Electromagnetic radiation in which two or more sets of waves have a constant phase relationship, i.e. with peaks and troughs always similarly spaced.

coherent units A system of *units of measurement in which derived units are obtained by multiplying or dividing base units without the use of numerical factors. *SI units form a coherent system; for example the unit of force is the newton, which is equal to 1 kilogram metre per second squared ($kg\,m\,s^{-2}$), the kilogram, metre, and second all being base units of the system.

coinage metals A group of three malleable ductile metals forming subgroup IB of the *periodic table: copper (Cu), silver (Ag), and gold (Au). Their outer electronic configurations have the form $nd^{10}(n+1)s^1$. Although this is similar to that of alkali metals, the coinage metals all have much higher ionization energies and higher (and positive) standard electrode potentials. Thus, they are much more difficult to oxidize and are more resistant to corrosion. In addition, the fact that they have *d*-electrons makes them show variable valency (Cu^I, Cu^{II}, and Cu^{III}; Ag^I and Ag^{II}; Au^I and Au^{III}) and form a wide range of coordination compounds. They are generally classified with the *transition elements.

coincidence circuit An electronic logic device that gives an output only if two input signals are fed to it simultaneously or within a specified time of each other. A *coincidence counter* is an electronic counter incorporating such a device.

coke A form of carbon made by the destructive distillation of coal. Coke is used for blast-furnaces and other metallurgical and chemical processes requiring a source of carbon. Lower-grade cokes, made by heating the coal to a lower temperature, are used as smokeless fuels for domestic heating.

colchicine An *alkaloid derived from the autumn crocus, *Colchicum autumnale*. It inhibits *spindle formation in cells, so preventing their division and inducing multiple sets of

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Dithiothreitol, a New Protective Reagent for SH Groups*

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Because of its low redox potential (−0.33 volts at *p*H 7), dithiothreitol (and its isomer, dithioerythritol) is capable of maintaining monothiols completely in the reduced state and of reducing disulfides quantitatively. Since this compound is a highly water-soluble solid with little odor and little tendency to be oxidized directly by air, it should prove much superior to the thiols now used as protective reagents for sulfhydryl groups.

Thiol groups such as those of coenzyme A and of some enzymes are readily oxidized in air to disulfides. To maintain these groups in the reduced state, another thiol such as cysteine, glutathione, mercaptoethanol, 2,3-dimercaptopropanol, or thioglycolate is often added so that interchange takes place according to reactions (1) and (2):

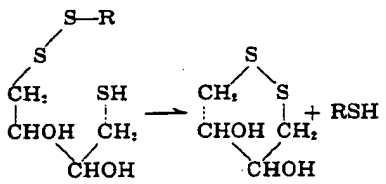
$$\text{CoA—SS—CoA} + \text{RSH} \rightleftharpoons \text{CoA—SH} + \text{R—SS—CoA} \quad (1)$$

$$\text{R—SS—CoA} + \text{RSH} \rightleftharpoons \text{R—SS—R} + \text{CoA—SH} \quad (2)$$

However, the equilibrium constants of these reactions are near unity, so that a sizable excess of the second thiol must be used. It occurred to this author that if reaction (2) were intramolecular and RSSR were a sterically favorable cyclic disulfide, there would be two products produced from one reactant, so that the equilibrium should be displaced to the right, particularly in dilute solutions. It appeared that a 1,4-dithiolbutane structure would produce the most sterically favorable cyclic disulfide, and that addition of hydroxy groups on the middle carbons should make the compound water soluble and reduce the stench of the thiol groups.

Dithiothreitol (DTT)[1] and dithioerythritol (DTE), the threo and erythro isomers of 2,3-dihydroxy-1,4-dithiolbutane, were therefore prepared as described by Evans *et al.* (1949) and found to have the desired properties. Reaction with a disulfide takes place according to reactions (3) and (4), and is complete in several minutes at *p*H 8.

$$\text{R—SS—R} + \text{HS—CH}_2(\text{CHOH})_2\text{CH}_2\text{—SH} \rightleftharpoons \text{RSH} + \text{R—SS—CH}_2(\text{CHOH})_2\text{CH}_2\text{—SH} \quad (3)$$



(4)

Attempts were made to determine the over-all equilibrium constant for reactions (3) and (4) by following the reduction of cystine by DTT or DTE, which can be conveniently measured because the thiol groups of DTT and DTE give only 4% as much color as cysteine in the nitroprusside assay of Grunert and Phillips (1951). Within experimental error, reaction between cystine and DTT or DTE went to completion, even when concentrations of the cyclic oxidized form of DTT or DTE (prepared by ferricyanide oxidation of DTT or DTE) ten times those of DTT or DTE were added.

The actual redox potential of DTT was measured by equilibrating the DTT-oxidized DTT system with the DPN^+-DPNH system in the presence of lipoamide and dihydrolipoic dehydrogenase, and measuring the amount of DPNH at equilibrium at 340 mμ (making suitable corrections for the absorption of lipoamide and oxidized DTT at this wavelength). The equilibrium constant for reaction of DTT with DPN^+ to give oxidized DTT and DPNH was about 2.5 at *p*H 7.0 and 35 at *p*H 8.1. Assuming the redox potential of DPN^+ to be −0.330 v at *p*H 7.0 (Burton and Wilson, 1953), the redox potential of DTT is −0.332 v at *p*H 7.0, and −0.366 v at *p*H 8.1. This is about 0.044 v more negative than the potential of lipoamide (Massey, 1960), corresponding to an equilibrium constant for reaction of lipoamide and DTT of 31.

* Supported in part by a grant (HE-05095) from the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used in this work: DTT, dithiothreitol; DTE, dithioerythritol; oxidized DTT, cyclic disulfide of DTT (*trans*-4,5-dihydroxy-*o*-dithiane); oxidized DTE, cyclic disulfide of DTE (*cis*-4,5-dihydroxy-*o*-dithiane).

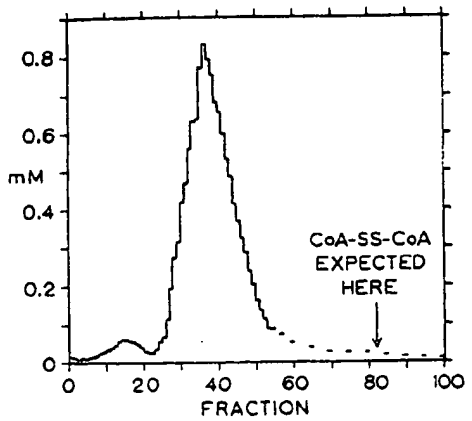


FIG. 1.—Chromatography of coenzyme A on diethylaminoethyl-cellulose-bicarbonate in the presence of DTT. A solution containing 135 μmoles of coenzyme A (Pabst) and 50 mg DTT in 200 ml was absorbed on a 1.8 × 20-cm column, and eluted with a 2-liter linear gradient of triethylammonium bicarbonate, *p*H 7.4, running from 0.1 to 1 M. All eluting solutions contained 0.1 mg/ml DTT. Fraction size, 10 ml. Concentrations are based on OD_{260}. No optical density was found in fractions 100–200.

Considerable uncertainty exists concerning the redox potentials of thiols such as cysteine, glutathione, and coenzyme A, with reported values at *p*H 7 ranging from −0.22 to −0.35 v (Clark, 1960). An attempt was made to determine the potential of the L-cysteine–L-cystine system by equilibration with the DPN^+–DPNH system in the presence of lipoamide and dihydrolipoic dehydrogenase. At *p*H 7.9 the equilibrium constant for the reduction of DPN^+ by cysteine was estimated to be 0.0013, which corresponds to a potential for cysteine of −0.21 v at *p*H 7.0. This value is close to that reported by Fruton and Clarke (1934) (−0.22), but more positive than the values obtained by others. Accepting this value, we can calculate an equilibrium constant of 1.3×10^4 for the reduction of cystine by DTT (reactions 3 and 4). Since the initial reaction of DTT and cystine (reaction 3) should have an equilibrium constant not far from unity, the equilibrium constant for the cyclization reaction (reaction 4) would be about 10^4. It is interesting to note that formation of the dithiane ring of oxidized DTT occurs more readily than formation of the dithiolane ring of lipoamide, as shown by the equilibrium constant of 31 for reduction of lipoamide by DTT.

The ability of DTT to keep a monothiol reduced can be seen from Figure 1, which shows the results of chromatography of commercial coenzyme A (Pabst) on diethylaminoethyl-cellulose-bicarbonate in the presence of DTT. Only one small ultraviolet-absorbing impurity is present, and there is no oxidized coenzyme A peak at all (commercial coenzyme A is only 75% reduced). Chromatography of the same preparation of coenzyme A in the absence of a reducing agent gives several small peaks (presumably mixed disulfides) in addition to the major peaks of reduced and oxidized coenzyme A.

In addition to the favorable equilibrium for reduction of disulfides, DTT and DTE possess other convenient properties. Both the reduced and oxidized forms are solids which are quite soluble in water and alcohols. Water solutions of the reduced forms are surprisingly stable to air oxidation (more stable than glutathione, for example), but their oxidation is catalyzed by the presence of a monothiol such as cysteine. The amount of DTT or DTE present can be determined by assaying

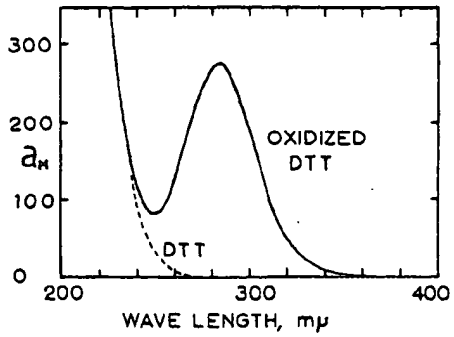


FIG. 2.—Ultraviolet spectrum of oxidized DTT and DTT in water.

for thiol groups both by a method which determines all thiol groups, and by the nitroprusside method (Grunert and Phillips, 1951), in which DTT and DTE give low color yields. The amount of the oxidized forms present can be determined from their ultraviolet spectrum (Fig. 2). Although the solid reduced forms and their concentrated solutions have the characteristic thiol odor, this is apparent only at close range, and these compounds do not need to be used in a hood.

DTT and DTE thus seem to do very admirably the job they were designed to do. Because of their low redox potential and other convenient properties, they are obviously the reagents of choice for protecting thiol groups. So far no real difference between the isomers has been noted, and the more easily prepared DTT has been routinely used in this laboratory.

EXPERIMENTAL

Dithiothreitol and Dithioerythritol.—The tetraacetyl derivatives of DTT and DTE were prepared by oxidation of *trans*-1,4-dibromobutene-2 to the corresponding dibromoglycol, acetylation, and reaction with potassium thioacetate as described by Evans *et al.* (1949). DTT and DTE were prepared from the tetraacetates by refluxing in 1 N methanolic HCl under N_2 for 5 hours, taking to dryness in a rotary flash evaporator, and storage over P_2O_5 and KOH *in vacuo* for several days. DTT prepared in this way melted at 40° (reported by Evans *et al.*, 1949, 43°) and was 97–100% pure by assay for SH groups. It can be sublimed at 37° (0.005 mm) onto a cold finger for further purification. DTE recrystallized from ether-hexane, mp 83° (reported by Evans *et al.*, 1949, 83°), was 100% pure by SH assay. Both DTT and DTE give full color yield when assayed for SH groups using *N*-ethyl maleimide (Roberts and Rouser, 1958; Alexander, 1958) or 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman, 1959), but only 4% of the color given by cysteine in the nitroprusside reaction (Grunert and Phillips, 1951).

Oxidized DTT and DTE.—The previously unreported cyclic disulfides of DTT and DTE (the *trans* and *cis* isomers, respectively, of 4,5-dihydroxy-*o*-dithiane) were prepared by ferricyanide oxidation. A solution of 1 g (6.5 mmoles) of DTT in 25 ml water was titrated with 0.8 M ferricyanide (with the *p*H kept at 7 by addition of 2 N KOH) until a yellow color persisted. Exactly 13 mmoles each of ferricyanide and base were required. The solution was evaporated to 10 ml and 200 ml ethanol was added. After filtration the clear filtrate was taken to dryness, and the crude product was crystallized by addition of hexane to a solution in ethyl acetate. The final product, mp 132°, was obtained by sublimation at 80° (0.005 mm) onto a cold finger.

Anal. Calcd. for $C_4H_8O_2S_2$: C, 31.56; H, 5.30; S, 42.13. Found: C, 31.58; H, 5.27; S, 42.27.

Oxidized DTE prepared similarly had the same melting point as oxidized DTT, but a mixture of the two melted over the range 110–120°.

Anal. Found: C, 31.84; H, 5.35; S, 42.15.

The ultraviolet spectrum of oxidized DTT is shown in Figure 2. The maximum of the disulfide peak is at 283 mμ (a_M = 273). The spectrum of oxidized DTE is essentially the same.

Redox Potentials of DTT and Cysteine.—Reactions were carried out at room temperature in 3 ml volume in silica cuvets containing 0.2 M buffer (phosphate, *p*H 7.0, or Tris, *p*H 7.9 or 8.1), 0.18 mg/ml crystalline dihydrolipoic dehydrogenase (Massey, 1960) and from 0.4 to 1.2 mM lipoamide. Either DPN^+ or DPNH (0.13–0.2 mM) was present initially. For the DTT experiments, the combined concentration of DTT and oxidized DTT was kept constant at 6.7 mM, and ratios of DTT to oxidized DTT of 4:1, 1:1, and 1:4 were used. The optical density of all components except enzyme was read, the enzyme was added, and the optical density was followed until equilibrium was reached. The amount of dihydrolipoic dehydrogenase used catalyzed a nearly instantaneous equilibration of DPN^+ and lipoamide, so that approach to equilibrium in the over-all reaction was limited by thiol interchange, equilibrium being reached in 1 minute at *p*H 8, and 10 minutes at *p*H 7 with DTT. The measured change in optical density was considered as a first approximation to be caused by DPNH formation or disappearance; corrections were later made for the small optical density changes resulting from changes in the concentrations of lipoamide and oxidized DTT.

In order to observe DPN^+ reduction by cysteine, it was necessary to use 42 mM cysteine and no cystine present initially. The observed reduction of DPN^+ was small, but was completely reversed by addition of 0.83 mM cystine.

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The Enzymic Condensation of a Thiol Ester–Type Carboxyl-activated Acylamino Acid with an Amino Acid Amide to Form a Peptide*

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Papain is capable of catalyzing a transfer reaction in which the thiol ester group of benzoylglycine thiol ethyl ester is replaced by glycinamide. This is the first known example of a proteolytic enzyme catalyzing a replacement reaction involving a thiol ester–type activated amino acid carboxyl group. The effect of glycinamide concentration upon this reaction has been studied. These results indicate that a two-step reaction occurs. The first step, the hydrolysis of the thiol ester, is rate determining. The reaction shows a *p*H optimum at *p*H 7.0.

The first indication that proteolytic enzymes can effect the hydrolysis of a thiol ester was reported by Goldenberg *et al.* (1950). These investigators observed, upon incubating acetyl DL-phenylalanine thiol ester with chymotrypsin, the strong odor of ethyl mercaptan. Strecker *et al.* (1955) have shown that ox brain and ox liver contain thiolesterases. In 1956 in this laboratory it was demonstrated that the plant proteolytic enzyme papain possessed powerful thiolesterase activity (Johnston, 1956). Papain was shown to catalyze the hydrolysis of benzoylglycine thiol ethyl ester (BGTEE)[1] and benzoylglycine thiol isopropyl ester as well as benzoylglycine ethyl ester. Previously Bergmann *et al.* (1935) reported that benzoylglycinamide is an active substrate for papain.

Cysteine-activated papain is known to catalyze the transamidation reaction involving the replacement of the amide of benzoylglycinamide by ^{15}N ammonia introduced as isotopic diammonium hydrogen citrate or ammonia (Fruton, 1950). Papain also catalyzes the replacement of amide nitrogen of a number of acylamino acid amides by the —NHOH group of hydroxylamine or by amino acids to form peptides (Johnston *et al.*, 1950a,b; Fruton *et al.*, 1950).

The discovery of the thiolesterase action of this proteolytic enzyme suggested the possibility that the enzyme might be capable of catalyzing the transfer of the acyl group of a thiol ester to an amino compound to form a peptidelike structure. Previous to the work reported here, the catalysis by proteolytic enzymes of a transfer reaction of this general type has not been reported. In this report the papain-catalyzed synthesis of BGGA from BGTEE and glycinamide is described.

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[1] The following abbreviations will be used: BGTEE = benzoylglycine thiol ethyl ester; BGGA = benzoylglycylglycinamide; BG = benzoylglycine.